The transcription factor ETS-1 regulates angiotensin II-stimulated fibronectin production in mesangial cells

Ping Hua, Wenguang Feng, Gabriel Rezonzew, Phillip Chumley, and Edgar A. Jaimes. The transcription factor ETS-1 regulates angiotensin II-stimulated fibronectin production in mesangial cells. Am J Physiol Renal Physiol 302: F1418–F1429, 2012. First published February 22, 2012; doi:10.1152/ajprenal.00477.2011.—Angiotensin II (ANG II) produced as result of activation of the renin-angiotensin system (RAS) plays a critical role in the pathogenesis of chronic kidney disease via its hemodynamic effects on the renal microcirculation as well as by its nonhemodynamic actions including the production of extracellular matrix proteins such as fibronectin, a multifunctional extracellular matrix protein that plays a major role in cell adhesion and migration as well as in the development of glomerulosclerosis. ETS-1 is an important transcription factor essential for normal kidney development and glomerular integrity. We previously showed that ANG II increases ETS-1 expression and is required for fibronectin production in mesangial cells. In these studies, we determined that ANG II induces phosphorylation of ETS-1 via activation of the type 1 ANG II receptor and that Erk1/2 and Akt/PKB phosphorylation are required for these effects. In addition, we characterized the role of ETS-1 on the transcriptional activation of fibronectin production in mesangial cells. We determined that ETS-1 directly activates the fibronectin promoter and by utilizing gel shift assays and chromatin immunoprecipitation assays identified two different ETS-1 binding sites that promote the transcriptional activation of fibronectin in response to ANG II. In addition, we identified the essential role of CREB and its coactivator p300 on the transcriptional activation of fibronectin by ETS-1. These studies unveil novel mechanisms involved in RAS-induced production of the extracellular matrix protein fibronectin in mesangial cells and establish the role of the transcription factor ETS-1 as a direct mediator of these effects.

chronic kidney disease; renin-angiotensin system

MALADAPTIVE ACTIVATION of the renin-angiotensin system (RAS) is critical in the pathogenesis of chronic kidney disease (CKD) of different etiologies including hypertension and diabetes (24). Angiotensin II (ANG II) is produced as a result of RAS activation and is a potent systemic vasoconstrictor and modulator of the renal microcirculation (15). In addition, ANG II has important nonhemodynamic effects that have been implicated in the pathogenesis of CKD, by promoting mesangial cell proliferation and increasing extracellular matrix (ECM) deposition (16).

Fibronectin is a multifunctional ECM protein that plays an important role in cell adhesion and migration related to fundamental processes such as embryogenesis (9), malignancy (33), wound healing (8), and maintenance of tissue integrity (22). Fibronectin also plays a major role in the development of matrix expansion and glomerulosclerosis characteristic of CKD (5). Through their binding to signal-transmitting integrin receptors, fibronectin can also modulate multiple cellular functions including cell adhesion, migration and chemotaxis, proliferation, differentiation, and apoptosis (32).

ETS-1 belongs to the ETS family of transcription factors that share a conserved ETS domain that recognizes the core consensus DNA sequence GGAA/T of target genes (28). ETS-1 is important in embryogenesis, angiogenesis, and cancer growth (6, 14, 20) and is essential for normal kidney development and the maintenance of glomerular integrity (2). We previously demonstrated that ANG II increases the glomerular expression of ETS-1 in vivo as well as in vitro in rat mesangial cells (29). We also showed that ETS-1 knockdown significantly reduces the mesangial cell expression of fibronectin in response to ANG II stimulation suggesting an important role for ETS-1 as a mediator of the profibrotic effects of ANG II (29). Given the important role of ANG II-mediated increases in ECM production in the pathogenesis of progressive CKD (46), we performed a series of experiments to test the hypothesis that ETS-1 directly activates the production of the ECM protein fibronectin in response to ANG II in rat mesangial cells.

MATERIALS AND METHODS

Materials. Human ANG II and antibody for β-actin were purchased from Sigma (St. Louis, MO). Candesartan, a specific ANG II type 1 receptor (AT₁) blocker, was a kind gift from AstraZeneca (Wilmington, DE). The epithelial growth factor receptor (EGFR) inhibitor AG 1478, the MEK inhibitor PD98059, the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the PI3K kinase inhibitor wortmannin were purchased from Calbiochem (La Jolla, CA). Antibodies for fibronectin, ETS-1, p300, Erk1/2, and p-Erk1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for phospho-ETS-1[pT38] was from Invitrogen (Camarillo, CA). Antibodies for SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), phospho-Akt, Akt, phospho-CREB, and cAMP response element binding (CREB) were purchased from Cell Signaling Technology (Danvers, MA). Peroxisome-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). DMEM low-glucose medium was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). ANG II dominant negative (ETS-1 DN) peptide and ETS-1 mutant (ETS-1 MU) peptides were synthesized by CPC Scientific (San Jose, CA) according to the sequences published by Oettgen et al. (27).

Cell culture. Rat mesangial cells line (CRL-2573) was purchased from America Type Culture Collection (Rockville, MD). The cells were grown in DMEM low glucose with 10% heat-inactivated FBS in the presence of 0.4 mg/ml G418 and antibiotic/antifungal solution and kept in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were grown to 60–70% confluence and arrested in DMEM low glucose with 0.5% FBS for 24 h before treatment.

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Plasmids. Firefly luciferase reporter vector (pGL3/basic) and promoter-less plasmid with Renilla luciferase (pRL-null) were purchased from Promega (Madison, WI). Mammalian expression vector pCMV-TAG was purchased from Stratagene (La Jolla, CA). A plasmid (pGL2/F1900) containing a rat fibronectin promoter fragment corresponding to nucleotides −1900 to +136 was kindly provided by Dr. In-San Kim (Dept. of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Daegu, Korea). A series of 5′ deletion constructs (nt −328/+136, −943/+136, −1900/+136) of the fibronectin promoter were made by PCR and restriction enzyme digestion and subcloned into pGL3 basic reporter vector. Mutations of transcription factor binding sites on the fibronectin promoter were generated with a QuickChange XL Site-Directed Mutagenesis kit from Stratagene. For ETS-1 binding sites at −773 and −237, the core sequence of GGAA was mutated to ATCT. For c-AMP-responsive element at −165, the core sequence of TGACGTC was mutated to TCACCTCA. A plasmid (pCMV-SPORT6) containing mouse ETS-1 cDNA was purchased from Open Biosystems (Huntsville, AL). The cDNA sequence encoding the mouse wild-type ETS-1 and the ETS-1 DNA-binding domain corresponding to amino acids 306–423 were cloned by PCR amplification and ligated in frame with a mammalian expression vector (pCMV-TAG5A). All PCR products were verified by sequencing (UAB Comprehensive Cancer Center, DNA Sequencing Core, University of Alabama at Birmingham).

Cell transfection and reporter gene assays. Rat mesangial cells were seeded in 24-well plates at a density of 5×10⁴ cells/well. Transient transfection was performed using TurboFect from Fermentas (Glen Burnie, MD) according to the manufacturer’s protocol. Renilla luciferase vector pRL-null was used to normalize transfection efficiency. Transfection mixtures contained a ratio of luciferase reporter construct to renilla of 10:1. Cotransfection with an expression plasmid, the ratios of DNA were 5:5:1, luciferase reporter: expression plasmid:renilla, respectively. Twenty-four hours after transfection, Firefly and Renilla luciferase activities were measured in a tube luminometer (SIRIUS Luminometer, Berthold Detection Systems, Pforzheim, Germany) using the Dual Luciferase System (Promega).

Nuclear extracts preparation and gel shift assays. Nuclear extracts were prepared by using a Universal Magnetic Co-IP kit (Active Motif, Carlsbad, CA) with modifications. Briefly, rat mesangial cells grown on a 100-mm plate were washed twice with ice-cold PBS. Then, 1 ml of ice-cold PBS was added to the dish and the cells were collected by gentle scraping with a cell lifter and transferred to a prechilled tube. Cell suspensions were centrifuged for 5 min at 500 g at 4°C. Cell pellets were then resuspended in freshly prepared hypotonic buffer and incubated on ice for 15 min. After being mixed with detergent, the cell suspensions were centrifuged at 14,000 g for 30 s at 4°C. The nuclear pellets were resuspended in freshly prepared digestion buffer and incubated for 10 min in a 37°C water bath. After centrifuging for 10 min at 14,000 g at 4°C, the supernatants were collected and stored at −80°C. Protein concentrations were measured using the DC protein assay reagents (Bio-Rad, Hercules, CA).

Gel shift assays were performed using a DIG Gel Shift Kit from Roche Applied Science (Roche, Germany). DNA oligos corresponding to the ETS-1 binding sites on the fibronectin promoter were synthesized with HPLC purification by Invitrogen. Complementary oligonucleotides were annealed and labeled with DIG-11-ddUTP by using the terminal deoxynucleotidyl transferase method following the kit protocol. For the binding reaction, 0.8 ng of labeled probe and 3–6 μg of nuclear extract from each sample were used. For competition experiments, 100- to 250-fold molar excess of unlabeled DNA oligos were added to the binding mixture for 5 min before the addition of labeled probe. For the supershift assay, 8–10 μg of ETS-1 antibody were preincubated with the nuclear extracts for 30 min at room temperature before the addition of labeled probe. DNA-protein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gel in 0.5× Tris/Borate/EDTA buffer for 50 min at 100 V. The DNA-protein complexes were then transferred to the positively charged nylon membrane by electrophoresing at 100 V for 50 min. The membranes were cross-linked at 120 mJ with a UV crosslinker (FisherBiotech, Phoenix, AZ). After being incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase, the DNA-protein binding complexes were detected with a chemiluminescent detection solution.

Western blot and chromatin immunoprecipitation. Western blotting and chromatin immunoprecipitation (ChIP) were performed using the ChIP-IT express Enzymatic kit from Active Motif, according to the manufacturer’s instructions. Briefly, cells were grown to 70% confluence on 150-mm dishes and were fixed in 1% formaldehyde for 5 min at room temperature. Cells were collected and incubated in ice-cold lysis buffer for 30 min. Cells were then homogenized gently in a dounce homogenizer to release the nuclei. After centrifugation, the nuclei pellets were saved and enzymatically sheared for 8 min. The sheared DNA was purified, quantitated, and immunoprecipitated with respective antibody using Magnetic Beads. A rabbit IgG antibody from Jackson ImmunoResearch Laboratories was used as control. After reverse cross-linking, the immunoprecipitated DNA and input DNA were assayed by real-time PCR and the products were analyzed as signal-to-noise ratio of ΔCt of input to samples. The primers corresponding to the two ETS-1 binding site regions on fibronectin promoter were as follows: for ETS-1 binding site 1 (EBS1), forward: 5′-ACC ACA GGC CCTT CTT AAA GAA AC-3′; reverse: 5′-CCC TCT CCT GAG CTT CAG C-3′; for ETS-1 binding site 2 (EBS2), forward: 5′-CTC CGA GTA TTC ACT TTA CAG C-3′, reverse: 5′-GTC CGC AAC AAA AGA GAT G-3′.

Statistical analysis. All data are presented as means ± SE. For statistical comparison of two groups, unpaired Student’s t-test was used. For statistical comparisons of more than two groups, ANOVA (StatView, BrainPower, Calabasas, CA) was used. Significance was considered when P < 0.05.

RESULTS

Erk1/2 and PI3K/Akt mediate phosphorylation of ETS1 in response to ANG II. We previously demonstrated that ANG II transcriptionally regulates the expression of ETS-1 in cultured mesangial cells (29). The transcriptional activity of ETS factors can be further modulated through posttranslational modi-
fications such as threonine-38 phosphorylation (43). In these experiments, we determined whether ANG II induces ETS-1 phosphorylation and the role of AT1, EGFR, the MAPKS Erk1/2, JNK, p38, and of PI3K/Akt on these effects.

For these experiments, we first measured ETS-1 threonine-38 phosphorylation in response to ANG II in rat mesangial cells. As shown in Fig. 1A, stimulation with ANG II for 10 min at a concentration range of $10^{-9}$ to $10^{-5}$ M resulted in a significant increase in ETS-1 phosphorylation at threonine-38. Time-dependent experiments showed a significant increase in ETS-1 phosphorylation that peaked between 10 and 15 min after treatment (Fig. 1B). Pretreatment with the AT1 blocker candesartan ($10^{-6}$ to $10^{-7}$ M) significantly prevented the ETS-1 phosphorylation induced by ANG II, demonstrating that these effects are mediated via activation of the AT1 (Fig. 1C).

Transactivation of the EGFR plays an important role in several of the actions mediated by ANG II in different tissues including the glomerular mesangium (30). To establish the role of the EGFR on ETS-1 phosphorylation, we determined the effects of EGFR inhibition on ANG II-mediated ETS-1 phosphorylation. As shown in Fig. 2A, EGFR inhibition with AG1478 and in a dose-dependent manner prevented ANG II-induced ETS-1 phosphorylation as well as Erk1/2 phosphorylation (Fig. 2B). We then proceeded to investigate whether MAPKS and the PI3K/Akt pathway participate in ETS-1 phosphorylation by ANG II. Figure 3A illustrates that treatment with ANG II at $10^{-6}$ M resulted in a time-dependent increase in ETS-1 phosphorylation. Pretreatment with the MEK inhibitor PD98059, the p38 MAPK inhibitor SB203580, or the JNK inhibitor SP600125 significantly inhibited ANG II-induced

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**Fig. 1.** ANG II induces ETS-1 phosphorylation in rat mesangial cells. Sixty to seventy percent of confluent rat mesangial cells were starved in 0.5% DMEM medium overnight and treated with ANG II at a final concentration range of $10^{-9}$ to $10^{-5}$ M for 10 min. ETS-1 phosphorylation and total ETS-1 expression were assessed by Western blot. The densitometric analysis of the ratio of p-ETS-1 to total-ETS-1 is presented as arbitrary OD units. A: ANG II significantly increased ETS-1 phosphorylation in all the concentrations tested compared with vehicle control ($*P < 0.05$ vs. control, $n = 3$). B: time-dependent effect of ETS-1 phosphorylation induced by ANG II ($*P < 0.05$ vs. control, $n = 3$). C: ANG II-induced ETS-1 phosphorylation was prevented by AT1 receptor blocker candesartan ($*P < 0.05$ vs. control, $#P < 0.05$ vs. ANG II, $n = 3$).
ETS-1 phosphorylation. However, the MEK inhibitor PD98059 produced a more complete inhibition of ETS-1 phosphorylation compared with the p38 and JNK inhibitors (Fig. 3A). Treatment with ANG II resulted in strong Erk1/2 phosphorylation and a more modest phosphorylation of p38 and JNK (Fig. 3, B, C, and D). MEK inhibition completely prevented Erk1/2 phosphorylation, while p38 and JNK inhibition partially reduced Erk1/2 phosphorylation. These findings suggest that the effects of p38 and JNK inhibition on ETS-1 phosphorylation could be mediated at least in part via reductions in Erk1/2 phosphorylation. To determine the role of PI3K/Akt on ETS-1 phosphorylation, rat mesangial cells were pretreated with the PI3K inhibitor wortmannin (10^{-6} M). As shown in Fig. 4, A and B, pretreatment with wortmannin significantly reduced ETS-1 and Akt phosphorylation demonstrating that PI3K/Akt is required for ETS-1 activation.

**ETS-1 mediates ANG II-induced fibronectin expression**. ETS-1 is an important regulator of ANG II-induced vascular inflammation and remodeling (49). In previous studies, we demonstrated that ANG II induces the expression of ETS-1 in cultured rat mesangial cells (29) and that ETS-1 siRNA knockdown significantly reduces the expression of fibronectin in these cells (29). To further study the role of ETS-1 on these effects, an ETS-1 dominant negative peptide was used to inhibit the function of ETS-1 as previously described (27). As shown in Fig. 5A, ANG II increased the expression of fibronectin in a time-dependent fashion in rat mesangial cells, which was prevented in a dose-dependent manner by pretreatment with the ETS-1 dominant negative peptide but not by a mutant ETS-1 peptide (Fig. 5B). Furthermore, transfection of rat MCs with a dominant negative form of an ETS-1 plasmid lacking the ETS-1 transcription domain also significantly inhibited fibronectin expression induced by ANG II compared with cells transfected with an empty vector (Fig. 5C). In the aggregate, these findings demonstrate that the transcription factor ETS-1 is essential for the profibrotic effects of ANG II in mesangial cells.

**ETS-1 regulates ANG II-induced fibronectin expression at a transcriptional level**. Previous studies showed that several transcription factors including NF-κB (19), AP-1 (4), CREB (25), and SP-1 (25) participate in the transcriptional regulation of the fibronectin gene. However, whether ETS-1 directly regulates the expression of the fibronectin gene in response to ANG II is not known. Using the MatInspector software (Genomatix, Ann Arbor, MI) to analyze the rat fibronectin promoter sequence up to nt 1908, several putative ETS-1 binding sites were identified. We therefore made a series of 5’ deletion constructs of the rat fibronectin promoter to identify the promoter regions in the fibronectin gene that are transactivated by ETS-1 in response to ANG II. As shown in Fig. 6 all three fibronectin promoter reporter constructs FN/329, FN/943, and FN/1900 were functional in rat mesangial cells, and ANG II significantly increased the activity of these promoter constructs by 50, 70, and 25%, respectively.

**Identification of binding sites for ETS-1 in the fibronectin promoter**. To verify the direct binding of ETS-1 to the promoter region nt −943/+136, which showed the highest fibronectin promoter activity, a gel shift assay was performed in rat mesangial cells treated with ANG II for 0, 2, and 4 h. Two oligonucleotides nt −777/−748 and nt −243/−214, which cover two putative ETS-1 binding sites core sequence, were synthesized and designated as EBS1 and EBS2, respectively (Fig. 7A). As shown in Fig. 7B, nuclear extracts from rat mesangial cells treated with ANG II formed a complex with the
labeled EBS1 (lanes 2, 3, 4). Competition with cold wild-type EBS1 dose dependently eliminated the observed complex (lanes 5, 6, 8). Incubation with an ETS-1 antibody partially inhibited the binding between the nuclear extract and labeled probe (lane 10), whereas a control antibody did not (lane 9), indicating the specificity of the protein binding to EBS1 by ETS-1. A similar experiment was conducted using labeled EBS2 as a probe. As shown in Fig. 7C, treatment with ANG

Fig. 3. Erk1/2 is required for ANG II-induced ETS-1 phosphorylation. Sixty to seventy percent of confluent rat MCs were serum-starved overnight and pretreated with the MEK inhibitor PD98059 (10^-6 M), the p38 MAPK inhibitor SB203580 (10^-6 M), or the JNK inhibitor SP600125 (10^-6 M) for 30 min before treatment with ANG II (10^-6 M) for the indicated time points. A: ANG II induced ETS-1 phosphorylation in time-dependent manner, which was significantly reduced by the 3 MAPK inhibitors (*P < 0.05 vs. control, #P < 0.05 vs. ANG II, n = 3). B: ANG II time dependently induced Erk1/2 phosphorylation, which was completely inhibited by PD98059 and SB203580 and partially by the JNK inhibitor SP600125 (*P < 0.05 vs. control, #P < 0.05 vs. ANG II, n = 3). C: ANG II significantly induced phosphorylation of p38 MAPK in a time-dependent manner (*P < 0.05 vs. control, n = 3). D: ANG II induced a small increase in JNK phosphorylation, which was significant after 10 min of ANG II but not at earlier time points (*P < 0.05 vs. control, n = 3).
II for 2 h (lane 3) resulted in a marked increase in binding of the nuclear extract to the labeled EBS2 that was reduced after 4 h of treatment (lane 4). Competition with cold wild-type EBS2 almost completely abolished the complex formation (lane 5). Supershift assay showed that the ETS-1 antibody partially replaced the binding between the nuclear protein and the probe (lane 7), whereas both control antibodies had no effect (lane 8, 9).

To confirm the binding of ETS-1 to specific ETS sites on the fibronectin promoter, a ChIP assay was performed to analyze the interaction of ETS-1 with the EBS1 and EBS2 binding sites in mesangial cells. PCR primers were designed to flank the EBS1 and EBS2 regions of the fibronectin promoter. Cross-linked chromatin were immunoprecipitated with a specific ETS-1 antibody or an isotype-matched control antibody and real-time PCR was performed on the immunoprecipitated chromatin after reversed cross-linking. As shown in Fig. 8, A and B, ANG II stimulation resulted in increased and specific ETS-1 binding to both EBS1 and to EBS2. In summary, these findings demonstrate that ETS-1 binds to two different binding sites in the fibronectin promoter.

ETE-1 increases the functional activity of the fibronectin promoter. To define the functional significance of the identified ETS-1 binding sites on the fibronectin promoter, site-directed mutagenesis was performed on the ETS-1 binding core sequences. Luciferase reporter constructs containing mutated EBS1 or EBS2 were transfected into rat mesangial cells. As shown in Fig. 9 (open bars), mutation of EBS1 (core sequence GGAA mutated to ATCT from nt 773 to nt 770) and EBS2 (core sequence GGAA mutated to ATCT from nt 237 to nt 234) decreased baseline promoter activity by 32.5 and 37.5%, respectively, compared with the wild-type construct nt 943.). Double mutation of both EBS1 and EBS2 did not result in further reductions in promoter activity.

We then performed cotransfection of each promoter construct with an ETS-1 mammalian expression vector. Cotransfection of an ETS-1 mammalian expression vector significantly increased luciferase expression by 55, 37, and 247%, respectively, with EBS1, EBS2, and both EBS1 and EBS2. The observed increases in fibroinectin promoter activity mediated by exogenous ETS-1 were significantly reduced by EBS1, EBS2, or double mutation (Fig. 9, filled bars).

To identify the ETS-1 binding sites responsible for the expression of reporter gene under the stimulation of ANG II, promoter constructs FN/H11002, FN/H11001, FN/H11002/EBS1, FN/H11002/EBS2, and FN/H11002/EBS1/EBS2 were transfected into rat mesangial cells. Six hours after transfection, cell medium was changed to 0.5% DMEM overnight and the cells were treated with ANG II (10^{-6} M) for 24 h and cells were collected for reporter gene assays. As shown in Fig. 10, treatment with ANG II resulted in a significant increase in luciferase activity in cells transfected with the wild-type promoter construct FN/H11002. Mutation of EBS1 resulted in a modest reduction in luciferase activity. However, when EBS2 or both EBS1 and EBS2 were mutated, the promoter lost the responsiveness to ANG II completely, indicating that EBS2 is the critical ETS-1 binding site responsible for the transactivation of the fibronectin promoter in response to ANG II.

CREB and its coactivator p300 are required for ETS-1-mediated upregulation of fibronectin. The transcription factor CREB and its coactivator p300/CBP are needed for ETS-1-mediated activation of the fibronectin promoter, as we performed experiments in which cotransfection of the fibronectin promoter and ETS-1 mammalian expression vector were accompanied by mutation of the cAMP response element (CRE) (core sequence TGACGTCA mutated to TCACCTCA from nt 159 to nt 159) while leaving EBS1 and EBS2 intact. This cotransfection resulted in a 63.5% reduction in promoter activity compared with the wild-type construct nt 943/+ 136, thus indicating that the cAMP-responsive element is critical in the transcriptional activation of the fibronectin promoter by ETS-1 (Fig. 9).

Based on these results, we performed a series of experiments to characterize whether ETS-1, CREB, and p300 directly interact with each other as assessed by Co-IP. Rat mesangial cells were treated with ANG II or vehicle for 15 min, and the cytoplasmic and nuclear proteins were extracted. As shown in Fig. 11A, treatment with ANG II resulted in a significant increase in phosphorylated ETS-1 and CREB as assessed by Western blot. The expression of these three transcription factors was predominantly nuclear. Nuclear extracts equivalent to 500–800 μg of protein were then immunoprecipitated with a rabbit polyclonal antibody against the N-terminal region of ANG II stimulation resulted in increased and specific ETS-1 mediated upregulation of fibronectin. The transcription factor CREB and its coactivator p300 are needed for ETS-1-mediated activation of the fibronectin promoter, as we performed experiments in which cotransfection of the fibronectin promoter and ETS-1 mammalian expression vector were accompanied by mutation of the cAMP response element (CRE) (core sequence TGACGTCA mutated to TCACCTCA from nt 159 to nt 159) while leaving EBS1 and EBS2 intact. This cotransfection resulted in a 63.5% reduction in promoter activity compared with the wild-type construct nt 943/+ 136, thus indicating that the cAMP-responsive element is critical in the transcriptional activation of the fibronectin promoter by ETS-1 (Fig. 9).

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ETS-1, a rabbit polyclonal antibody against the coactivator p300, or the same amount of a nonspecific IgG from the same species as a negative control. The immunoprecipitated proteins were loaded on a 7.5% SDS polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and probed with an anti-phospho-CREB antibody. As shown in Fig. 11B, probing with an anti-pCREB antibody recognized a band in the anti-p300 immunoprecipitates (lane 1) and in the anti-ETS-1 immunoprecipitates (lane 2) with an approximate molecular weight of 43 kDa. This band was almost absent in extracts from cells treated with vehicle (lane 3), and in control IgG immunoprecipitates (lane 4). These findings therefore demonstrate the interaction between ETS-1, CREB, and its coactivator p300. As additional controls, the same immunoprecipitates were probed with an anti-ETS-1 antibody or IgG control. As shown in Fig. 11B, there was strong ETS-1 expression in samples immunoprecipitated with ETS-1 antibody (lanes 2 and 3), lower in samples immunoprecipitated with p300 antibody (lane 1), and absent in samples immunoprecipitated with IgG (lane 4). ANG II treatment caused a marked increase in ETS-1 interaction with CREB that peaked after 15 min (Fig. 11C). As expected, all samples showed strong bands when the immunoprecipitates were probed with IgG control.

To further determine whether ETS-1 also binds to p300, nuclear protein extracts from ANG II- or vehicle-treated rat mesangial cells were immunoprecipitated with either an anti-ETS-1 antibody or a nonspecific IgG from the same species. As shown in Fig. 11D, an anti-p300 antibody recognized a band that corresponds to 300 kDa in the anti-ETS-1 immunoprecipitates, but not in control IgG immunoprecipitates, indicating that ETS-1 forms a complex with p300. As control the same membrane was stripped and immunoblotted with an anti-ETS-1 antibody and IgG. As shown in Fig. 11D, samples immunoprecipitated with ETS-1 showed a strong band corresponding to ETS-1 while in IgG-immunoprecipitated samples ETS-1 was undetectable. As expected, all samples showed strong bands when the immunoprecipitates were probed with IgG control.

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DISCUSSION

The role of the RAS in the pathophysiology of progressive kidney disease is uncontroversial (24). Numerous experimental studies have demonstrated a key role for ANG II in the pathogenesis of CKD and multiple clinical trials have demonstrated that blockade of RAS improves clinical outcomes in patients with CKD (1).

In addition to its hemodynamic effects in the glomerular microcirculation (11), a key component in the progression of renal disease, ANG II induces medial hyperplasia of small renal artery vessels, proliferation of glomerular cells, increased glomerular production of fibronectin, and tubulointerstitial injury (15). ANG II mediates these effects via several intracellular signals including reactive oxygen species (ROS), PKC, PIK3/Akt, and MAPKS (12) that ultimately result in the activation of transcription factors that promote the expression of profibrotic and proinflammatory cytokines and chemokines (16, 32).

ETS-1 is a member of ETS family of transcription factors that share similar DNA-binding domain. It involves a wide variety of functions and activities in vasculogenesis (44), angiogenesis (10), hematopoiesis (21), neuronal development (42), and carcinogenesis (39). The renal expression of ETS-1 is increased in a variety of models of renal injury. In the anti-Thy model of glomerulonephritis, ETS-1 expression was found to be increased especially in the glomerular mesangium and at a lesser degree in podocytes and glomerular endothelial cells (31). In an ischemic model of acute renal failure, the tubular expression of ETS-1 was found to be increased and associated with increased expression of cyclin D, suggesting a role for ETS-1 in the control of tubular regeneration in acute renal failure (38). In rats with anti-glomerular basement-induced glomerulonephritis, there is also increased upregulation of ETS-1 in the glomeruli and in the interstitium (26). Moreover, we previously demonstrated that ANG II increases the expression of the transcription factor ETS-1 both in cultured rat

Fig. 7. Direct binding of ETS-1 to putative ETS-1 binding sites on FN promoter region –943/+136. A: schematic representation of the FN promoter fragment +136/–943 with 2 putative ETS-1 binding sites designated as EBS1 and EBS2. B: gel shift assays demonstrated binding of ETS-1 to EBS1 (lanes 2, 3, 4, and 7) that was prevented by an excess of exogenous unlabeled oligos (lanes 5, 6, and 8) and partially prevented by an anti-ETS-1 antibody (lane 10) but not by IgG control (lane 9). C: gel shift assays demonstrated binding of ETS-1 to EBS2 (lanes 2, 3, 4, and 6) that was prevented by an excess of exogenous unlabeled oligos (lane 5) and anti-ETS-1 antibody (lane 7) but not by IgG control (lane 8) or anti-CREB antibody (lane 9).
mesangial cells and in the kidney cortex of rats infused with ANG II (29). We also demonstrated that these effects were mediated by ANG II-induced generation of ROS and that silencing of ETS-1 reduces the production of fibronectin in response to ANG II (29). In a model of balloon injury, we also showed that ETS-1 mediates the formation of neointima and regulates the expression of several adhesion molecules and proinflammatory cytokines (7).

Previous studies showed that phosphorylation of ETS-1 at a threonine residue (T38) increases the binding ability of this transcription factor to target promoters (35, 36, 47). Our studies demonstrate that ANG II induces ETS-1 phosphorylation via activation of the AT1. Transactivation of the EGFR after stimulation with ANG II and AT1 activation plays a major role on several biological effects of ANG II including the

Fig. 8. Chromatin immunoprecipitation (ChIP) analysis of ETS-1 binding to FN promoter in ANG II-stimulated rat MCs. Cross-linked chromatin prepared from rat MCs treated with ANG II or vehicle was immunoprecipitated with ETS-1 antibody or control IgG. Primers were designed to flank EBS1 and EBS2 in the FN promoter. Real-time PCR was performed using input chromatin or immunoprecipitated chromatin. A: PCR products were visualized by 1.3% agarose gel electrophoresis and GelRed staining. B: data are shown as signal:noise ratio against input over background. Stimulation with ANG II resulted in significant increases in ETS-1 binding to both EBS1 and EBS2 (n = 3 by duplicate, *P < 0.05 vs. control).

Fig. 9. Mutational analysis of ETS-1 binding sites (EBS) on FN promoter. Site-directed mutagenesis of EBS or cAMP response element (CRE) on FN promoter was used to construct mutant reporter plasmids for EBS1, EBS2, EBS1/EBS2, and CRE. Cells were cotransfected with ETS-1 expression vector or empty vector as control. Relative luciferase activity was calculated as a ratio of firefly luciferase activity to Renilla luciferase activity. Mutation of EBS1 (FN/−943/EBS1), EBS2 (FN/−943/EBS2), both EBS1 and EBS2 (FN/−943/EBS1/EBS2), or CRE (FN/−943/CRE) reduced luciferase expression comparing with wild-type (WT) construct (open bar). Cotransfection with an ETS-1 mammalian expression vector increased luciferase expression driven by the WT reporter construct (FN/−943) and reduced by cotransfection with the EBS1, EBS2, EBS1/EBS2, and CRE mutants [filled bar; n = 3 by duplicate, *P < 0.05 vs. WT construct (FN/−943), #P < 0.05 vs. cotransfection with ETS-1 and WT constructs].

Fig. 10. Identification of EBS on FN promoter regions −943/+136 that transactivate reporter gene expression in response to ANG II. Mesangial cells at 60–70% confluence were transiently transfected with promoter constructs FN/−943, FN/−943/EBS1, FN/−943/EBS2, and FN/−943/EBS1/EBS2. Six hours after transfection, cell medium was changed to 0.5% DMEM overnight and cells were treated with ANG II (10⁻⁶ M) or vehicle for 24 h. Cell lysates were collected for reporter gene assay. ANG II-induced transactivation of each promoter construct is expressed as fold induction of relative luciferase activity. The mutation of EBS1 resulted in a small reduction in relative luciferase activity comparing with WT (P = 0.07) while mutation of EBS2 resulted in a significant reduction in relative luciferase activity (n = 3, *P < 0.05 vs. control, #P < 0.05 vs. WT).
production of ROS and activation of growth-promoting pathways (40, 41). Our studies utilizing a specific EGFR inhibitor demonstrated that EGFR participates on ETS-1 phosphorylation. Several lines of evidence suggest an important role for MAPKS in mediating the growth-promoting effects of ANG II in mesangial cells (12). In addition, ANG II can induce the activation of the serine-threonine kinase Akt/protein kinase B (PKB) in mesangial cells and represent a critical signaling pathway that mediates protein synthesis and mesangial cell hypertrophy in response to ANG II (48). In these studies, we demonstrated that Erk1/2 and Akt/PKB phosphorylation and at a much lower degree p38 or JNK are required for ETS-1 phosphorylation in mesangial cells in response to ANG II. The pharmacologic inhibition of p38 or JNK also resulted in partial reduction of Erk1/2 phosphorylation, suggesting either nonspecific inhibition of Erk1/2 or the presence of cross talk between these MAPKs. In addition, we demonstrated that EGFR transactivation is also required for ERK1/2 phosphorylation after ANG II treatment.

Fibronectin is an important component of the fibroproliferative response triggered in tissues after injury and plays a major role in the development of matrix expansion and glomerulosclerosis (5). In addition, fibronectin affects cellular processes involved in inflammation and tissue repair (8, 9, 33). Through their binding to signal-transmitting integrin receptors, fibronectin can modulate multiple cellular functions including cell adhesion, migration and chemotaxis, proliferation, differentiation, and apoptosis (32). Furthermore, fibronectin and other matrix molecules activate monocyte/macrophage cell lineages and stimulate their production of proinflammatory mediators including prostaglandins and cytokines such as IL-1 (34), TNF (3), and IL-6 (45) among others.

In these studies, we hypothesized that ETS-1 can directly activate the expression of ECM proteins such as fibronectin in

Fig. 11. Transcriptional factor cAMP response element binding (CREB) and its coactivator p300 are involved in ETS-1 upregulation of ANG II-induced FN expression. Rat MCs at a 60–70% confluence were serum-starved in 0.5% DMEM overnight and treated with ANG II at 10^{-6} M or vehicle for 15 min. A: ANG II increased expression of phosphorylated form of transcription factor ETS-1 and CREB in rat MCs nuclear fraction. B: nuclear extracts prepared from ANG II- or vehicle-treated cells were immunoprecipitated with antibodies against p300, ETS-1, or IgG and immunoblotted with anti-phospho-CREB antibody. Both p300 and ETS-1 immunoprecipitates positively immunoblotted with CREB suggesting a direct interaction between ETS-1, CREB, and the coactivator p300. C: overnight serum-starved MCs were treated with ANG II at indicated times. Nuclear extracts were immunoprecipitated with ETS-1 antibody and immunoblotted with anti-phospho-CREB antibody. The interaction between ETS-1 and CREB peaked after 15 min of ANG II treatment. D: nuclear extracts prepared from ANG II- or vehicle-treated MCs were immunoprecipitated with ETS-1 or IgG and immunoblotted with anti-p300 antibody. ETS-1 directly interacted with p300. All the coimmunoprecipitation figures are representative of at least 3 independent experiments.

Fig. 12. Schematic diagram of ANG II-induced ETS-1 phosphorylation and FN transcriptional regulation by ETS-1 in rat MCs. ANG II-induced ETS-1 phosphorylation requires AT1 receptor activation and transactivation of the endogenous EGFR, which stimulate MAPKS, predominantly Erk1/2, and activate the PI3K/Akt pathway, resulting in subsequent phosphorylation of ETS-1. Once phosphorylated, ETS-1 binds to specific binding sites on the FN promoter. The transcription initiation of ETS-1 needs the participation of p300 and CREB, which bridges ETS-1 with the transcription apparatus of FN.
the glomerular mesangium. First and by using an ETS-1 dominant negative peptide and a dominant negative form of an ETS-1 plasmid lacking the transcription domain (27, 29), we confirmed the critical role of ETS-1 as a mediator of ANG II-stimulated generation of fibronectin in mesangial cells. Utilizing promoter gene assays, we identified a fibronectin promoter fragment (−934/+136) with the maximal promoter activity in response to ANG II. Two putative ETS-1 binding sites at (−777/−748) and (−243/−214) on this fragment −934/+136 were then studied to characterize the role of ETS-1 on fibronectin gene expression. Both by gel shift assay and by ChIP assay we made the novel observation that ETS-1 binds directly to these two binding sites on the (−934/+136) fibronectin promoter fragment. Mutagenesis of the two ETS-1 binding sites showed that promoter activity was reduced by about one-third when either site was mutated, and double mutation of the sites did not further reduce the promoter activity. However, when a CRE at −165/−159 was mutated, 63.5% of the promoter activity was lost indicating the importance of CREB in ETS-1-regulated fibronectin transcription. CREB is a versatile phosphorylation-dependent transcription factor involved in diverse pathological conditions (13). Several different serine-threonine kinases have been shown to promote phosphorylation of CREB at its transcription activating site, serine 133. Once serine 133 of CREB is phosphorylated, CREB interacts with its coactivator protein, CREB-binding protein (CBP), or p300 that promote gene transcription by adapting DNA-binding transcription factors with basal transcription machinery, and integrating transcription factors and chromatin through acetylation (13, 17). CREB has been shown to be involved in a variety of cellular processes, including cell proliferation, adaptive responses, and glucose homeostasis among others (23). In mesangial cells stimulated with ANG II and transfected with wild-type or mutated constructs of the fibronectin promoter, we determined that, although ETS-1 binds to both EBS1 and EBS2 as determined by EMSA and ChIP, most of the transcriptional activity of ETS-1 on the fibronectin promoter is mediated by binding to the EBS2.

Since mutation of the CRE at −165 abolished almost two-thirds of fibronectin promoter activity, we performed a series of Co-IP experiments to determine whether CREB was involved in the transcriptional activation of the rat fibronectin promoter by ETS-1. As shown in Fig. 8, we demonstrated protein-protein interactions not only between ETS-1 and CREB but also between ETS-1 and p300. Although these studies were focused on the role of ETS-1 on the transcriptional activation of fibronectin, we hypothesize that ETS-1 also regulates other genes involved in the proinflammatory effects of ANG II. The modulation of these effects by targeting a common transcriptional mediator such as ETS-1 may have important implications in the development of strategies aimed at modulating the proinflammatory effects of ANG II.

In conclusion, we investigated the molecular mechanisms in the regulation of ANG II-induced fibronectin expression by the transcription factor ETS-1 in rat mesangial cells. Our studies demonstrate that ETS-1 is one of the transcription factors that transactivate the expression of fibronectin induced by ANG II. This transactivation occurs through the interaction of ETS-1 with CREB and p300 (Fig. 12). In addition, the phosphorylation of ETS-1 induced by ANG II involves both Erk-MAPK pathway and PI3K/Akt pathway (Fig. 9). Finally, our studies support that transcription factors activated in response to mal-adaptive activation of RAS may be a potential target for the treatment or prevention of CKD of different etiologies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.H., W.F., G.R., and P.C. performed experiments; P.H., W.F., P.C., and E.A.J. analyzed data; P.H. and E.A.J. interpreted results of experiments; P.H. and E.A.J. prepared figures; P.H. and E.A.J. drafted manuscript; P.H., W.F., G.R., P.C., and E.A.J. approved final version of manuscript; E.A.J. conception and design of research; E.A.J. edited and revised manuscript.

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ETS-1 stimulates fibronectin in mesangial cells